Ruminal micro-organisms do not adapt to increase utilization of poly-phenol oxidase protected red clover protein and glycerol-based lipid

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Abstract

BACKGROUND: The enzyme polyphenol oxidase (PPO) reduces the extent of proteolysis and lipolysis within red clover fed to ruminants with subsequent increases in the efficiency of N utilization and the level of beneficial polyunsaturated fatty acids in their products (meat and milk). It has also been reported that red clover feeding alters the rumen microbial population compared to grass feeding. This study investigated whether the observed shifts in the microbial population of the rumen when ruminants are fed red clover silage (RC) as opposed to grass silage (G) represented an adaptation by the micro-organisms to increase the utilization of PPO-protected protein and glycerol-based lipid.

RESULTS: The experiment consisted of two periods where ruminally fistulated dairy cows were offered either RC or G for 2 weeks, followed by collection of rumen fluid, which was then used in *in vitro* incubations to investigate lipolysis and proteolysis over time in plant material derived from red clover plants with either wild type PPO expression (PPO+) or PPO expression reduced to undetectable levels by gene silencing (PPO−). Proteolysis and lipolysis ($P < 0.05$) were lower after 24 h of incubation in the PPO+ treatment than the PPO− treatment irrespective of rumen fluid. Biohydrogenation of C18 polyunsaturated fatty acids was also lower on the PPO+ treatment than the PPO− treatment, with no effect of rumen fluid.

CONCLUSION: These results suggest that microbial changes to red clover feeding did not result in an increased ability of the micro-organisms in the present study to utilize either PPO-protected protein or glycerol-based lipid.

Keywords: polyphenol oxidase; red clover silage; grass silage; proteolysis; lipolysis

INTRODUCTION

The enzyme polyphenol oxidase (PPO) is a copper metalloprotein that catalyses hydroxylation of monophenols to *ortho*-diphenols and oxidation of *ortho*-diphenols to *ortho*-quinones.[1] The PPO-generated *ortho*-quinones are highly reactive electrophilic molecules that covalently modify and crosslink a variety of nucleophilic cellular constituents, such as proteins, amines and amides, leading to formation of melanin pigments associated with the 'browning reaction' of fruit and vegetables.[2] These addition products may be further oxidized to their respective quinones and a second addition may occur, resulting in formation of crosslinked protein complexes which are resistant to enzymatic digestion by mammalian proteases, including trypsin, α-chymotrypsin and pepsin.[3] The reduction in protein digestion through this mechanism has been shown to increase flow of dietary N to the small intestine, with the potential to increase dietary N-use efficiency and final product quality.[4]

Lee *et al*.[5] have shown that the PPO enzyme can reduce the extent of both plant-mediated proteolysis and lipolysis in red clover (*Trifolium pratense* L.) in a simulated rumen environment. They hypothesized that this may be due to complexing of phenols with plant proteins and polar membrane lipid and/or the denaturing of plant proteases and lipases, for reduction in proteolysis and lipolysis, respectively. Lipolysis in the rumen is a prerequisite for microbial hydrogenation (i.e., biohydrogenation) of polyunsaturated fatty acids (PUFA). This addition of hydrogen to PUFA in the rumen leads to an increase in the saturated fat content of ruminant products and is considered to reduce the healthiness of ruminant fat to the humans that consume it.[6] A reduction in ruminal hydrogenation occurred in steers fed red clover silage diets[7,8] and this has been followed through to an enhanced polyunsaturated nature of the milk and beef muscle product.[9]

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(*Received 9 April 2008; revised version received 10 July 2008; accepted 12 July 2008*)
Published online 9 September 2008; DOI: 10.1002/jsfa.3366

 J Sci Food Agric 0022–5142/2008/$30.00

Huws *et al.*[10] indicated that red clover silage feeding as opposed to grass silage feeding resulted in a significant change in the rumen microbial ecosystem within a 2-week period. Such changes could represent adaptation of the rumen microbial population to increase utilization of PPO-protected protein and glycerol-based lipid. This study investigated whether such microbial changes in rumen fluid from cows offered red clover silage will result in higher levels of proteolysis and lipolysis in *in vitro* incubations of red clover tissue from plants with either active or genetically silenced PPO1 genes[11] than rumen fluid from cows offered grass silage.

MATERIALS AND METHODS

Experimental design and aim

The experiment consisted of two periods where two ruminally fistulated dairy cows were offered either red clover (cv. Milvus) silage or grass (*Lolium perenne* cv. AberElan) silage for 2 weeks, followed by collection of rumen fluid and diet change-over for the second period. The two rumen inocula from cows on grass or red clover silage were then used in *in vitro* incubations to investigate lipolysis and proteolysis over time for red clover tissue derived from plants with either wild type levels of PPO or PPO reduced to undetectable levels by RNA interference. The resulting data were used to assess the effect of rumen microbial adaptation to red clover on the PPO protection mechanism of protein and glycerol-based lipid.

Plants, inoculum and treatments

Plant material used for the proteolysis and lipolysis studies was derived from a cross between a clone having normal levels of foliar PPO activity derived from WI-2 red clover germplasm[12] and NRC27-64-1, a red clover plant having undetectable levels of PPO activity due to transformation with a pHannibal-based gene-silencing construct.[11] The resulting seeds were sown in individual pots containing John Innes No. 2 loam-based compost and inoculated with rhizobium culture 2 days later. The plants were grown and maintained under controlled conditions (temperature of 20/15 °C, photoperiod of 16 h, and humidity of 0.6 kPa at both temperatures). Presence or absence of the silencing transgene was determined by standard polymerase chain reaction (PCR) techniques using the primers 5′-AGTTGGGAAATTGGGTTCGAAATCG-3′ and 5′-TCATTAAAGCAGGACTCTAGAGGATC-3′, which anneal to the pdk intron (sense) and OCS terminator (antisense) regions of the pHannibal vector to amplify the antisense arm of the construct. The PPO phenotype of individual plants was determined by the quantitative assay described below. Four plants lacking the transgene and having wild type levels of PPO activity were designated as PPO+, whereas nine plants containing the transgene and having undetectable levels of PPO activity were designated as PPO−.

For the *in vitro* studies, the PPO+ and PPO− red clover plants were on a 6-week regrowth before harvesting at 5 cm above soil level to give *ca* 200 g fresh weight (FW) of each. Harvested herbage was crushed with a rolling pin, cut into 5 mm strips, left on a laboratory bench for 1 h and split into two equal quantities to provide material for *in vitro* incubations in period 1 and 2 before being frozen and retained at −20 °C (1 and 3 weeks for periods 1 and 2, respectively). During each period the respective material was defrosted and a sample (50 g FW) of each red clover (PPO+ and PPO−) taken to determine freeze-dry matter, PPO activity and chemical characteristics. The remaining material was then weighed into 32 tubes: 16 PPO+ and 16 PPO− (2.5 g FW per tube). This provided quadruplicate tubes at each time point (0, 2, 6, and 24 h). The day before the weighing out of the forage, anaerobic incubation medium was made up as described by Goering and Van Soest[13] and placed in a water bath at 39 °C overnight with continual CO_2 purging. Rumen fluid was collected from two rumen fistulated cows, which were allocated to either red clover silage or grass silage at random and fed *ad libitum* for 2 weeks. Hand-squeezed rumen fluid (1 L) was collected from each cow at the end of each period and transferred back to the laboratory in a temperature-regulated flask (39 °C). The cows were then offered the alternative silage for the second period. For each period anaerobic buffer (7.5 mL) was dispensed into each of the 32 extraction tubes (containing either the PPO+ or PPO− red clover tissue) by peristaltic pump, gassing the headspace with CO_2 as the tubes were sealed. Each tube was reduced with 0.35 mL of reducing agent[13] and inoculated with 2.5 mL of the appropriate strained rumen fluid, coming either from red clover fed (RC) or grass silage fed (G) dairy cows.

Incubations and lipid analysis

During each period the tubes were incubated in the dark at 39 °C and a set of four tubes harvested at 0, 2, 6 and 24 h for each treatment. At these time points the supernatant was subsampled for ammonia-N (1 mL mixed with 100 µL of 2 mol L^{-1} HCl and maintained at −20 °C) and free amino acid (FAA) analyses (1 mL maintained at −20 °C). The incubation tubes were then frozen with liquid N_2 and the contents freeze-dried. Once freeze-dried the lipid was extracted by adding 4 mL chloroform–methanol (2:1, v/v) and 100 µl internal standard (C21:0 15 mg mL^{-1} $CHCl_3$). This was centrifuged at 2000 × *g* and the solvent layer transferred to a clean tube before the addition of a further 4 mL of chloroform–methanol (2:1, v/v), which was vortexed and recentrifuged as before, pooling the solvent layer with the previous extract in the clean tube. This was repeated once more so that three solvent layers were pooled. The pooled extract was split in two, with half transferred to a clean tube to undergo lipid fractionation by thin layer chromatography (TLC),[14]

J Sci Food Agric **88**:2479–2485 (2008)
DOI: 10.1002/jsfa

which separated the lipid into four fractions: membrane lipid (ML, phospho- and galacto-lipid), triacylglycerol (TAG), diacylglycerol + monoacylglycerol (DAG + MAG) and FFA. Each collected fraction was extracted from the TLC plates and converted into fatty acid methyl esters (FAME) by the addition of 4 mL of 0.5 mol L^{-1} HCl in methanol and 2 mL toluene containing 0.4 mg mL^{-1} C23:0 as an internal standard and heating for 2 h at 50 °C. The remaining sample, not used for TLC, was dried down under N_2 at 50 °C resuspended in 1 mL heptane and converted to FAME using the bimethylation procedure (1.4 mol L^{-1} HCl in methanol and 0.5 mol L^{-1} NaOH in methanol) of Kramer and Zhou[15] to give total fatty acid content of each sample, which was used to calculate biohydrogenation of the C18 PUFA. FAME were analysed by gas–liquid chromatography on a CP Sil 88 FAME column (100 m × 0.25 mm i.d., Chrompack UK Ltd, London, UK) with split injection (30:1). Peaks were identified from standards (ME61, Larodan Fine Chemicals, Malmo, Sweden; S37, Supelco, Poole, UK) and quantified using the internal standard (C21:0 for total fatty acid analyses and C23:0 for lipid fractionation analyses).

Ammonia-N and free amino acid analysis

Ammonia-N was assessed colorimetrically using indophenol blue,[16] on a segmented flow analyser (ChemLab Instruments Ltd, Hornchurch, UK). Total FAA content was calculated using the ninhydrin method of Winters *et al*.[17]

Chemical analysis and PPO assay

Water-soluble carbohydrate (WSC) of the red clover was determined spectrophotometrically using anthrone in sulfuric acid on a Technicon Autoanalyser (Technicon Corporation, New York, USA).[18] Ash and by mass difference organic matter (OM) were analysed by combusting the ground samples at 550 °C for 6 h in a muffle furnace. Total nitrogen was determined by micro-Kjeldahl technique using 'Kjeltec' equipment (Perstorp Analytical Ltd, Maidenhead, UK). Neutral detergent fibre (NDF) was determined as described by Van Soest *et al*.[19] and acid detergent fibre (ADF) was analysed according to the method of Van Soest and Wine[20] using the Tecator Fibretec System equipment (Tecator Ltd, Thornbury, Bristol, UK). For the PPO activity assay, plant tissue was extracted according to the method of Winters and Minchin[21] and assayed according to the method of Robert *et al*.[22] In brief, leaf material (*ca* 0.5 g FW) was extracted at 0 °C in 2 mL McIlvaine buffer (pH 7) containing 0.1 mol L^{-1} ascorbic acid to inhibit PPO activity. Extracts were centrifuged at 15 000 × *g* for 10 min at 4 °C and the supernatant retained. Supernatants were desalted by applying to columns (1.5 × 6 cm) containing bio-Gel P6DG (Biorad, Hemel Hempstead, UK) prepared in McIlvaine buffer (pH 7) and centrifuging at 2500 × *g* for 6 min at 4 °C. Active PPO content was determined spectrophotometrically at 420 nm using 10 μL of eluted fraction with 15 μL 0.001 mmol L^{-1} copper sulfate, 10 mmol L^{-1} methylcatechol and 1.5 mL McIlvaine buffer. Total PPO activity (active + latent) was calculated with the addition of 0.25% sodium dodecyl sulfate in the McIlvaine buffer. One unit of enzyme (U) was defined as the amount of enzyme that produced 1 μmol of quinone per minute based on the absorption at λ 420 nm of a known concentration of quinones formed through the reaction of methylcatechol and sodium periodate,[23] giving a conversion factor of U = 2.717 × Δ optical density.

Calculations and statistical analysis

Lipolysis was calculated as the proportional loss of ML and not as total net loss of esterified lipid across all classes (DAG + MAG + TAG). This approach is used in 'living' tissue where enzymes such as acylCoA:*sn*-1,2-diacylglycerol acyltransferase (DAGAT) upregulate during periods of stress and convert DAG and MAG released during the breakdown of ML into TAG.[5] Therefore an equation which takes into account the net loss of all esterified lipid will underestimate considerably the lipolytic action of the tissue in degrading ML. This approach with 'living' tissue has been reported previously[5] and is calculated according to the equation below:

$$\text{Lipolysis} = (\text{ML}_{T0} - \text{ML}_{Tx})/\text{ML}_{T0}$$

where T = time; 0 = 0 h; x = 2, 6, or 24 h.

Lipolysis was analysed using a repeated-measures analysis of variance with red clover line (PPO+ *versus* PPO−) × rumen fluid (G *versus* RC) as the treatment blocking according to animal + period. The effect of animal was tested and found to be not significant ($P = 0.989$). Biohydrogenation was calculated according to the equation below:

$$\text{Biohydrogenation of C18 PUFA} = \left(\sum \text{C18 PUFA}_{T0} - \sum \text{C18 PUFA}_{Tx}\right)/\sum \text{C18 PUFA}_{T0}$$

where $\sum$C18 PUFA$_{T0}$ = sum of all C18 PUFA at time 0 h; $\sum$C18 PUFA$_{Tx}$ = sum of all C18 PUFA at time 2, 6 or 24 h.

Biohydrogenation was analysed statistically using a general analysis of variance with red clover line (PPO+ *versus* PPO−) × rumen fluid (G *versus* RC) as the treatment blocking according to animal + period. Ammonia-N production and release of FAA were used to predict proteolysis and were analysed as described for lipolysis. All statistical operations were performed with Genstat 8.1 (Lawes Agricultural Trust, Rothamstead, UK).[24]

RESULTS AND DISCUSSION

Chemical analyses and polyphenol oxidase activity

Chemical compositions of the wild type and transformed red clovers[11] were similar with: 178 and 168 g

DM kg^{-1} FW, 911 and 914 g OM kg^{-1} DM, 40.2 and 41.1 g N kg^{-1} DM, 296.4 and 287.0 g NDF kg^{-1} DM, 257.2 and 214.6 g ADF kg^{-1} DM, 77.4 and 78.1 g WSC kg^{-1} DM, 0.84 and 0.85 dry matter digestibility for PPO+ and PPO−, respectively. The PPO activity was significantly different between wild type and PPO-silenced red clover: 1950 and 0 U g DM^{-1} for total PPO (latent + active) and 135 and 0 U g DM^{-1} for active PPO for PPO+ and PPO−, respectively. The use of transgenic PPO-silenced material,[11,12] as opposed to studies with mutant lines with low PPO activities,[5,25,26] reduces the potential differences in isoenzyme activity and chemical composition between the two lines.

Proteolysis

The mechanism of PPO activity in reducing proteolysis seems likely to be that PPO-generated *ortho*-quinones from vacuolar plant *ortho*-diphenols react with nucleophilic sites on cellular proteins, such as sulfo-amino acids,[27] forming a quinone covalently bound to protein. Following further oxidation the diphenol is reformed resulting in the formation of phenol-bound protein. Proteolysis thus would be reduced either directly by active inhibition of proteases or a reduction in general protein solubility through protein-phenol-protein binding.[28] The phenol crosslinked proteins has been shown to be partially resistant to proteases of mammals (trypsin, α-chymotrypsin and pepsin),[3] silage micro-biota[26] and rumen micro-biota.[4,29,30] Huws *et al.*[10] showed significant shifts in the microbial ecology of the rumen when beef steers were switched from grass silage diets to red clover silage diets within 2 weeks of feeding. Such shifts may represent an increase in the ability of the rumen micro-biota to utilize phenol-bound protein. To date no account has been taken for potential adaptation of the rumen microbial ecosystem to PPO protection by comparing red clover adapted animals with naive animals to determine whether there are increases in utilization of the phenol-bound protein. Such adaptation could result in a reduced potency of the PPO protection mechanism. In the current study predictors of proteolysis in the form of ammonia-N and FAA liberation for the two red clovers in the two different rumen fluids are shown in Fig. 1. No differences were observed in either predictor up to 2 h, where after proteolysis for PPO+, red clover was significantly lower than PPO− in both rumen fluids, with no significant difference between origin of rumen fluid from RC or G. Table 1 summarizes the results after 24 h and shows that FAA and ammonia-N concentrations were significantly higher in incubations containing PPO− red clover irrespective of the rumen fluid. These results confirm the protection of protein by PPO in the presence of rumen micro-organisms.[4,29,30] They also indicate that shifts in rumen microbial ecology to red clover feeding[10] do not represent an adaptation to increase the utilization of PPO-protected protein and may indeed be a consequence of the increased supply of PPO protected

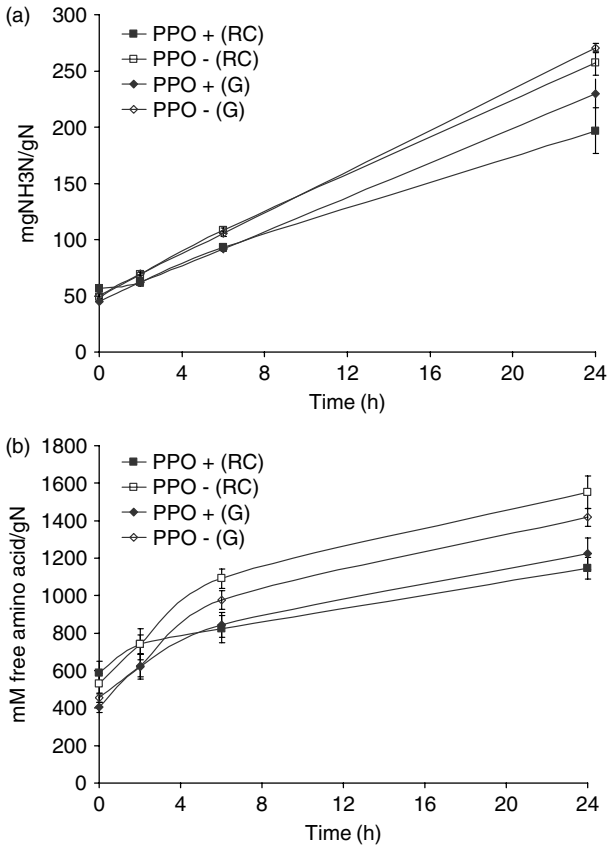

Figure 1. Predictors of proteolysis in red clover with (+) and without (−) PPO activity incubated for 24 h at 39 °C in the presence of rumen fluid from cows fed on either grass (G) or red clover (RC) silage: (a) Ammonia-N; (b) free amino acids.

protein. In the present study the protection response of the PPO+ treatment in reducing ammonia-N release (19%) was similar to that for FAA (19%), indicating that there was no further PPO-induced protection against microbial catabolism of the FAA once liberated. Whether this is true for all amino acids, especially sulfo-amino acids which are likely to be bound to phenol, requires further investigation in view of the consequent bio-availability of these sulfo-amino acids to animals fed red clover.

Lipolysis and C18 PUFA biohydrogenation

Proportional changes in the lipid fractions are shown in Fig. 2 and lipolysis for the two red clovers incubated in either RC or G-derived rumen fluids are shown in Fig. 3. FFA shifts across the incubations followed the pattern for lipolysis, with a greater increase in FFA release on the PPO− treatments than with PPO+, with no difference between inocula other than at 24 h where G was higher than RC for both PPO treatments, suggesting a greater release of fatty acids with the G-adapted rumen fluid. Changes in DAG and TAG follow patterns previously described by Lee *et al.*[5] The initial rise in DAG is due to the cleaving of the polar head group of the ML, releasing DAG, which can then be acted upon by DAGAT, forming TAG. However, these fractions will fluctuate as this process of formation works in conjunction with processes of

J Sci Food Agric **88**:2479–2485 (2008)
DOI: 10.1002/jsfa

Table 1. Lipolysis, C18 PUFA biohydrogenation (g g^{-1}) and predictors of proteolysis for red clover with (+) and without (–) PPO activity incubated for 24 h at 39 °C in the presence of rumen fluid from cows fed on either grass (G) or red clover (RC) silage

	G		RC			P		
	+	–	+	–	SED	+ *v.* –	G *v.* RC	Int.
C18:2	0.55	0.61	0.55	0.60	0.046	NS	NS	NS
C18:3	0.78	0.84	0.72	0.82	0.039	*	NS	NS
Lipolysis	0.71	0.89	0.73	0.84	0.044	*	NS	NS
FAA (M g^{-1} N)	1.22	1.42	1.15	1.55	0.151	*	NS	NS
Ammonia-N (g g^{-1} N)	0.23	0.27	0.20	0.26	0.016	**	NS	NS

SED, standard error of the difference; + *v.* –, PPO effect; G *v.* RC, rumen fluid effect; Int., interaction effect; NS, not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$.

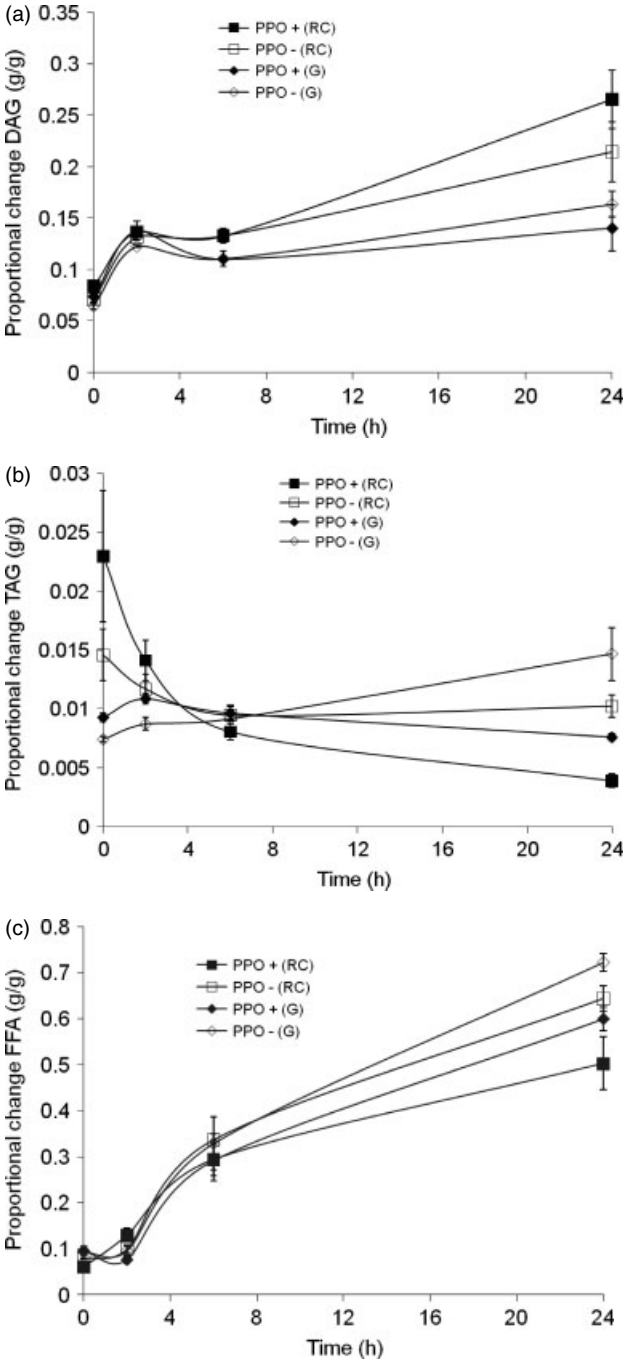

Figure 2. Changes in (a) DAG, (b) TAG and (c) FFA in red clover with (+) and without (–) PPO activity incubated for 24 h at 39 °C in the presence of rumen fluid from cows fed on either grass (G) or red clover (RC) silage.

degradation such as lipolytic acyl hydrolase, which randomly cleaves glycerol fatty acid ester bonds. For DAG at 6 and 24 h RC inoculum samples had higher levels than G inoculum, which may suggest that the cleaving of the polar head group was lower in G inoculum or indeed DAGAT activity was higher, as also suggested by the higher TAG levels for G compared to RC at 24 h. However, there is also a PPO effect in TAG at 24 h as PPO– is significantly higher than PPO+, which indicates a PPO retardation of DAGAT activity similar to its effect on plant lipase.[5,25]

Lipolysis was similar up to 6 h, after which the extent of lipolysis for the PPO+ plant material was significantly lower than the PPO– incubations, irrespective of rumen fluid. The protection of glycerol-based lipids against lipolysis by the action of PPO was first reported as a plant-mediated response,[5,31] suggesting a deactivation of lipase protein, although an inhibitory action of grape seed extract on lipases was previously reported.[32] Later work showed that lipid was also protected in the presence of rumen micro-organisms,[25] suggesting something other than deactivation of plant lipases. Further, since PPO cannot function in the anaerobic conditions of the rumen, deactivation of microbial lipases is unlikely. This alludes to an active protection mechanism of the glycerol-based lipid through the PPO-induced chemical cascade. Proposed protection mechanisms include lipid–phenol complex formation

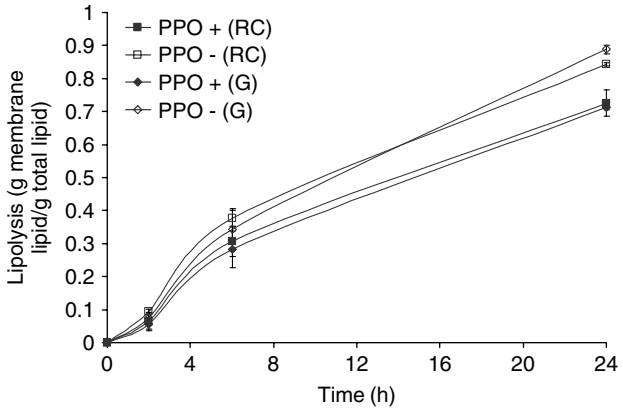

Figure 3. Lipolysis in red clover with (+) and without (–) PPO activity incubated for 24 h at 39 °C in the presence of rumen fluid from cows fed on either grass (G) or red clover (RC) silage.

either through polar head group binding or carboxyl binding[33] or, as recently suggested, lipid micelle formation within protein–phenol complexes.[30] The latter mechanism would explain the current results, as a lack of adaptation to increase utilization of the phenol-bound protein by the micro-organisms would also result in a retention of the PPO-induced protection of glycerol-based lipid. However, irrespective of the mechanism, the current results suggest that sustained feeding of red clover does not result in an adaptation of the rumen micro-organisms to increase lipolysis.

Table 1 summarizes the 24 h results for lipolysis and C18 PUFA biohydrogenation, and shows that lipolysis and C18:3 biohydrogenation were significantly higher in incubations containing PPO− red clover irrespective of the rumen fluid. As expected, with a reduction in lipolysis, a prerequisite for microbial hydrogenation of PUFA, biohydrogenation of C18:3 was significantly reduced by the action of PPO and there was a non-significant numerical reduction ($P < 0.1$) in C18:2 biohydrogenation. Similar responses have been observed when red clover silage has been fed to beef steers,[7,8] with a greater effect of PPO on the more unsaturated fatty acid. A greater protection of C18:3 than C18:2 is difficult to explain but may be related to its preferential incorporation into photosynthetic structures such as the chloroplasts,[34] which also contain the PPO enzyme.

Shifts in microbial populations have been shown to have significant effects on the biohydrogenation pathway when fish or marine oils have been fed.[35] However, in the current study the assumed induced microbial shift through red clover silage feeding did not result in changes in the extent of biohydrogenation compared with grass silage feeding and was only reduced as a result of the presence of PPO. This indicates that the active compound within red clover that reduces biohydrogenation is PPO and *ortho*-diphenol, PPO's substrate, and that it is not related to any shift in the microbial population induced when red clover is fed.

CONCLUSIONS

Although shifts occur in the microbial population as a consequence of red clover feeding, it does not result in a greater utilization of PPO-protected protein or glycerol-based lipid. The different inocula did result in slight changes in the fluctuations of different lipid fractions (DAG and TAG) but did not ultimately result in shifts in lipolysis of the ML. In addition, the active constituent of red clover in reducing C18 PUFA biohydrogenation is PPO and not a consequence of the microbial population shift, as with other dietary regimes (fish oil).

ACKNOWLEDGEMENTS

This work was jointly funded by the UK Department of Environment Food and Rural Affairs and the European Union as part of a framework VI project (PROSAFEBEEF) FOOD-CT-2006-36241. The authors would like to thank and acknowledge Martin Leyland and Naomi Ellis for the care of the animals at Trawsgoed Research Farm, Ana Winters for assistance with the PPO assays and Delma Jones and her staff for the analytical chemistry services.

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